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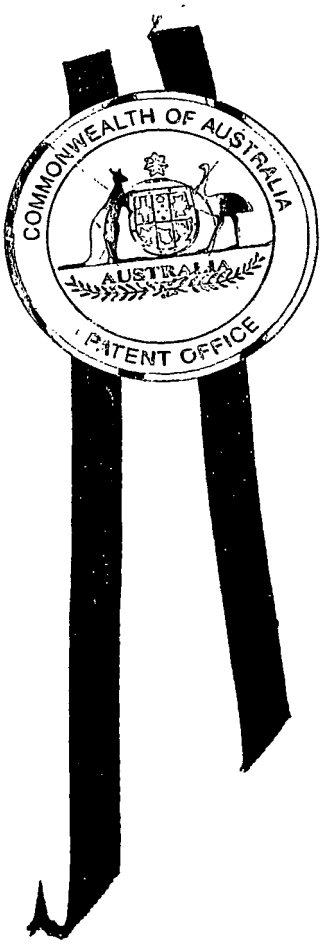

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Twenty-seventh day of September 2004

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Regulation 3.2

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PROVISIONAL SPECIFICATION

Invention Title: "HORMONE RECEPTOR GENES AND MIGRAINE SUSCEPTIBILITY"

The invention is described in the following statement:

TITLE

HORMONE RECEPTOR GENES AND MIGRAINE SUSCEPTIBILITY

FIELD OF THE INVENTION

THIS INVENTION relates to identifying a genetic predisposition to migraine. More particularly, this invention relates to identifying a polymorphism in a hormone receptor gene that predicts a predisposition to migraine and/or confirms clinical diagnosis of migraine.

BACKGROUND OF THE INVENTION

Migraine is a common disorder with variable expression [1]. The exact cause is unknown and there are no recognisable diagnostic pathological changes. Diagnosis is based on symptoms and their groupings. The lack of clear symptom definitions and precise diagnostic criteria, has led to variability in diagnosis. The International Headache Society [2], has however, recently prepared a new classification for headaches that has made diagnosis clearer and more precisely defined. This system uses the presence of specific attributes to establish diagnosis. The two main types of migraine are termed *migraine without aura*, previously known as common migraine, and *migraine with aura*, previously termed classical migraine. *Migraine without aura* is characterised by recurrent headache, lasting 4 - 72 hours, with at least two of the following attributes: unilateral location, pulsating quality, moderate to severe intensity and/or aggravation by physical activity. It is also associated with nausea and/or vomiting, or with photophobia and phonophobia. At least 5 attacks of headache fulfilling these criteria are required to separate this type of migraine from episodic tension-type headache. *Migraine with aura* is

characterised by neurological symptoms that usually precede or accompany headache. These symptoms develop over 5 - 20 minutes, and usually last less than 60 minutes. They most commonly include visual disorders, unilateral numbness or weakness, and aphasia or other speech disorders [3]. Headache, nausea, photophobia and/or phonophobia usually follow these symptoms, with headache lasting 4 - 72 hours. Over 80% of migraine sufferers, have headaches without neurological symptoms, while about 20% suffer from *migraine with aura*. There are a number of other less common types or sub-types of migraine that are accompanied by distinctive neurological symptoms. These include retinal migraine, in which unilateral visual disorders, which may involve temporary blindness, occur with or without headache; familial hemiplegic migraine (FHM), in which headache is accompanied by prolonged hemiparesis; and acephalgic migraine which can involve a variety of neurologic symptoms without headache.

The age of onset of migraine is varied. In females, the onset of disorder is usually at, or shortly after puberty, although many children are also diagnosed as suffering from migraine. Much less frequently, onset occurs in middle life and occasionally onset begins during menopause [4]. Once onset begins, the manifestation of the disorder may vary within a individual. The pattern and the clinical features of attacks can vary greatly with age in an individual and also between affected family members. It is not uncommon for an individual at different stages in life, to suffer from migraines that, based on clinical features, would be classified as different diagnostic types. Such variations can also be seen within members of the same family [5]. A recent

study indicated that 45% of *migraine with aura* families have *migraine without aura* cases [27]. In general, migraine attacks usually decrease in frequency and intensity with increasing age.

The pathogenesis and pathophysiology of migraine are poorly understood. Cerebral blood flow changes, specifically a decrease corresponding to the clinically affected area, have been noted as occurring before or at the onset of aura symptoms, in a number of sub-types of *migraine with aura*. In *migraine without aura*, however, regional cerebral blood flow remains normal or slightly increased.

Migraine is diagnosed in about 10% of adults but the disorder may be often undiagnosed and hence prevalence is likely to be higher [20]. Prevalence rates vary depending on migraine definition and population sampled. Kurtze [21] determined a conservative prevalence rate of 10% in the US, while Dalsgaard-Nielsen and Ulrich [22] found a Danish prevalence rate of 16-23%. More recent and comprehensive studies have indicated prevalence rates of 16% in the European general population [23] while in the US, prevalence was determined to be 4% in children, 6% in adult men and 18% in adult women [24]. A large Dutch survey revealed that the lifetime prevalence of migraine in women was 33% and the 1-year prevalence in women was 25%. In men, this study showed that the lifetime prevalence was 13.3% and the 1-year prevalence was 7.5% indicating that overall the prevalence of migraine may be even higher than previously reported [31].

Migraine shows strong familial aggregation. Approximately 50% of migraine sufferers have an affected first degree relative [6], with familial

incidence figures varying from 61% [22] to 90% [26] and of heritability estimates of 40% to 60% [32]. The mode of transmission of migraine is controversial but has generally been believed to be autosomal dominant with reduced penetrance [25]. Studies by Mochi *et al* [27] support a common genetic background for *migraine with* and *without aura* and indicate that there may be a major gene contributing to the disease. A recent review of migraine twin, spouse and family aggregation studies, strongly suggested that both sub-types of migraine are genetically determined with the mode of inheritance most likely multifactorial. However, autosomal dominant inheritance with reduced penetrance, could not be excluded in either sub-type of migraine [28].

SUMMARY OF THE INVENTION

The present invention is broadly directed to identification of a genetic predisposition to migraine according to the presence of a polymorphism in a hormone receptor gene such as a human estrogen receptor gene or a progesterone receptor gene.

In one aspect, the invention provides a method of identifying a predisposition to migraine including the step of identifying an isolated nucleic acid that comprises a nucleotide sequence of at least a fragment of a hormone receptor gene comprising a polymorphism that indicates said individual has an increased predisposition to migraine compared to an individual without the polymorphism.

In one embodiment, said nucleotide sequence is of at least a fragment of exon 8 of an estrogen receptor gene, wherein if said nucleotide sequence comprises a polymorphism encoding codon 594 of an estrogen receptor, said

individual has an increased predisposition to migraine compared to an individual without the polymorphism.

Suitably, the polymorphism is a guanine to adenine change at nucleotide 2014 of the estrogen receptor α (ERα) gene.

In another embodiment, said nucleotide sequence is of at least a fragment of a progesterone receptor gene, wherein if said nucleotide sequence comprises a 306 base pair insertion in intron G of said progesterone receptor gene, said individual has an increased predisposition to migraine compared to an individual without the polymorphism.

Suitably, said individual is a human.

In another aspect, the invention provides a kit for identifying a predisposition to migraine for use in the method of the first aspect, said kit comprising one or more primers, probes and, optionally, one or more other reagents for identifying said polymorphism.

Throughout this specification, unless the context requires otherwise, the words "comprise", "comprises" and "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

DETAILED DESCRIPTION OF THE INVENTION

Migraine is a painful and debilitating disorder that affects up to 18% of the population. It imposes a significant economic burden on society due to the costs of medical care, treatment and lost productivity. Genetic and environmental factors play a role in migraine susceptibility although the pathophysiological mechanisms are unclear. Steroid hormones have long been considered a triggering

factor involved in the onset of many migraine attacks. As the hormonal milieu in humans is influenced by hormone receptors, the present inventors reasoned that genetic variation in hormone receptor genes may effect migraine susceptibility.

This invention sets forth and confirms the hypothesis that one or more polymorphisms in hormone receptor genes, such as the estrogen receptor gene and progesterone receptor gene, may be associated with migraine susceptibility.

This candidate gene study involved a case-control design including DNA samples from 275 unrelated migraineurs and 275 unrelated controls who have been matched for age, sex and ethnicity. Polymorphisms in the progesterone receptor (PgR) gene, estrogen receptor (ERα) gene, and androgen receptor (AR) gene were analysed. Allele and genotype frequencies were compared between the groups by generating contingency tables and incorporating chi-squared statistical analyses. Significant findings were followed up with a family based association test on 174 nuclear pedigrees comprised of 607 individuals.

DNA was amplified using PCR techniques. Genotypes were determined for a 306 base pair insertion in the progesterone receptor gene (PROGINS), a trinucleotide repeat variant in the androgen receptor gene, and a restriction fragment length polymorphism in the estrogen receptor gene (exon 8 codon 594). The restriction fragment length polymorphism in the estrogen receptor gene (exon 8 codon 594) and the 306 base pair insertion in intron G of the progesterone receptor gene were genetic factors linked to migraine.

Throughout this specification "*predisposed to migraine*" means that an individual has an increased probability of suffering from migraine and includes situations where said individual is not yet exhibiting clinical symptoms of

migraine and where said individual is already displaying migraine symptoms. Furthermore, migraine includes "*migraine with aura*" and "*migraine without aura*" as hereinbefore described.

In one embodiment, the present invention provides for determination of a predisposition to migraine according to whether an individual has a polymorphism in an estrogen receptor allele that encodes residue 594 of a human estrogen receptor protein. Said polymorphism, if present, is in exon 8 of an estrogen receptor gene. It will therefore be appreciated that by isolating a nucleic acid corresponding to at least a fragment of exon 8 of an estrogen receptor gene that potentially includes the polymorphic codon, a determination can be made as to whether an individual is predisposed to migraine.

Suitably, the polymorphism is a guanine to adenine change at nucleotide 2014 of an estrogen receptor α (ERα) gene.

This is a "silent" polymorphism in that the encoded amino acid is not altered.

In another embodiment, present invention provides for determination of a predisposition to migraine according to whether an individual has a polymorphism in a progesterone receptor allele in the form of a 306 base pair insertion in intron G. It will therefore be appreciated that by isolating a nucleic acid corresponding to at least the portion of intron G that potentially includes the insertion, a determination can be made as to whether an individual is predisposed to migraine.

In the context of the present invention by "*corresponds to*" and "*corresponding to*" is meant that the isolated nucleic acid comprises the

nucleotide sequence information of at least a fragment of exon 8 of the estrogen receptor gene that includes codon 594 or the relevant portion of intron G or the progesterone receptor gene.

For the purposes of this invention, by "*isolated*" is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state. Isolated material may be in native or recombinant form.

By "*protein*" is meant an amino acid polymer. The amino acids may be natural or non-natural amino acids, D- or L- amino acids as are well understood in the art.

A "*peptide*" is a protein having less than fifty (50) amino acids.

A "*polypeptide*" is a protein having fifty (50) or more amino acids.

The term "*nucleic acid*" as used herein designates single-or double-stranded mRNA, RNA, cRNA and DNA inclusive of cDNA and genomic DNA and DNA-RNA hybrids.

The term "*gene*" is used herein as a discrete nucleic acid unit that may comprise one or more of introns, exons, open reading frames and regulatory sequences such as promoters and polyadenylation sequences.

The term "*polymorphism*" is used herein to indicate any nucleotide sequence variation in an allelic form of a gene that occurs in a human population.

This term encompasses mutation, insertion, deletion and other like terms that indicate specific types of polymorphisms.

A "*polynucleotide*" is a nucleic acid having eighty (80) or more contiguous nucleotides, while an "*oligonucleotide*" has less than eighty (80) contiguous nucleotides.

A "*probe*" may be a single or double-stranded oligonucleotide or polynucleotide, suitably labeled for the purpose of detecting complementary sequences in Northern or Southern blotting, for example.

A "*primer*" is usually a single-stranded oligonucleotide, preferably having 15-50 contiguous nucleotides, which is capable of annealing to a complementary nucleic acid "template" and being extended in a template-dependent fashion by the action of a DNA polymerase such as *Taq* polymerase, RNA-dependent DNA polymerase or Sequenase™.

The terms "*anneal*", "*hybridize*" and "*hybridization*" are used herein in relation to the formation of bimolecular complexes by base-pairing between complementary or partly-complementary nucleic acids in the sense commonly understood in the art. It should also be understood that these terms encompass base-pairing between modified purines and pyrimidines (for example, inosine, methylinosine and methyladenosine) and modified pyrimidines (for example thiouridine and methylcytosine) as well as between A,G,C,T and U purines and pyrimidines. Factors that influence hybridization such as temperature, ionic strength, duration and denaturing agents are well understood in the art, although a useful operational discussion of hybridization is provided in to Chapter 2 of

CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Eds. Ausubel et al. John Wiley & Sons NY, 2000), particularly at sections 2.9 and 2.10.

As used herein, a "*nucleic acid sequence amplification technique*" includes but is not limited to polymerase chain reaction (PCR) as for example described in Chapter 15 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.* (John Wiley & Sons NY USA 1995-2001) strand displacement amplification (SDA); rolling circle replication (RCR) as for example described in International Application WO 92/01813 and International Application WO 97/19193; nucleic acid sequence-based amplification (NASBA) as for example described by Sooknanan *et al.* 1994, Biotechniques 17 1077; ligase chain reaction (LCR) as for example described in International Application WO89/09385 and Chapter 15 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY *supra*; and Q-β replicase amplification as for example described by Tyagi *et al.* 1996, Proc. Natl. Acad. Sci. USA 93 5395.

As used herein, an "*amplification product*" refers to a nucleic acid product generated by any nucleic acid amplification technique.

Diagnostic methods

The present invention provides methods for determining whether an individual is predisposed to migraine.

Generally, the methods of the invention are nucleic acid-based methods, given that the hormone receptor polymorphisms described herein have been initially been identified and confirmed at the nucleic acid level.

Furthermore, the 594 codon polymorphism is silent with regard to the encoded alanine.

However, it is postulated that the 306 bp insertion in the progesterone receptor gene may affect protein expression, hence protein based methods of detection may be used according to the present invention.

Such methods are well known in the art and include western blotting, ELISA, two dimensional protein profiling, protein arrays, immunoprecipitation, radioimmunoassays and radioligand binding, although without limitation thereto.

With regard to nucleic acid detection, an isolated nucleic corresponding to at least a fragment of a hormone receptor gene is isolated from any appropriate source of nucleic acid, such as lymphocytes or any other nucleated cell type, preferably obtainable by a minimally-invasive method

Preferred diagnostic methods may employ nucleic acid sequence amplification techniques such as PCR.

In one example, PCR-based restriction fragment length polymorphism analysis may be used.

In another example PCR method that may also be useful is Bi-PASA (Bidirectional PCR Amplification of Specific Alleles), as for example described in Liu *et al.* 1997, Genome Res. 7 389-399.

Another potentially useful PCR method as allele-specification oligonucleotide hybridization, as for example described in Aitken et al., 1999, J Natl Cancer Inst 91 446-452.

It will also be well understood by the skilled person that identification of said polymorphism may be performed using any of a variety of techniques such as fluorescence-based melt curve analysis, SSCP analysis, denaturing gradient gel electrophoresis (DGGE) or direct sequencing of amplification products.

Melt curve analysis can be performed using fluorochrome-labeled allele-specific probes which form base-pair mismatches when annealing to wild-type DNA strands in heterozygotes. Alternatively, fluorescent DNA-intercalating dyes such as SYBR Green 1 can reveal the presence of these base-pair mismatches by virtue of their lower melting temperature (T_m) compared to fully complementary sequences. A useful example of allele-specific melt curve analysis can be found, for example, in International Publication No. WO97/46714.

DGGE also exploits T_m differences, but uses differential electrophoretic migration through gradient gels as a means of distinguishing subtle nucleotide sequence differences between alleles. Examples of DGGE methods can be found in Fodde & Losekoot, 1994, Hum. Mutat. 3 83-9 and United States Patents 5,045,450 and 5,190,856.

The or each polymorphism used according to the invention may also be identified by direct sequencing of a PCR amplification product, for example. An example of nucleic acid sequencing technology is provided in Chapter 7 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.* (John Wiley & Sons NY USA 1995-2001).

In yet another embodiment, a polymorphic hormone receptor-encoding nucleic acid linked to migraine may be identified by a microarray method of the invention.

Microarray technology has become well known in the art and examples of methods applicable to microarray technology are provided in Chapter 22 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.* (John Wiley & Sons NY USA 1995-2001).

With respect to the present invention, a preferred microarray format comprises a substrate such as a glass slide or chip having an immobilized, ordered grid of a plurality of nucleic acid molecules, such as cDNA molecules, although without limitation thereto.

A microarray would typically comprise a nucleic acid having said estrogen receptor gene polymorphism and/or a nucleic acid having said progesterone receptor gene polymorphism together with control estrogen receptor and progesterone receptor nucleic acids.

Such a microarray could also include a plurality of other nucleic acids indicative of other diseases that have an underlying genetic basis and be useful in large scale genetic screening, for example.

So that the present invention may be more readily understood and put into practical effect, the skilled person is referred to the following non-limiting examples.

EXAMPLES

Introduction

ERα is located on chromosome 6q25.1. It is over 140 kilobases in size and has 8 exons (Iwase *et al.*, 1996). ERα is expressed in various human brain regions including the hypothalamus, limbic system, hippocampus, cortices of the temporal lobe and the brainstem (Osterlund *et al*, 2000). It is expressed in serotonin neurons of some species (Bethea *et al*, 2002). In addition to alternate splicing mechanisms, different promoters are used to regulate ERα in distinct neuronal populations (Osterlund *et al*, 2000). Along with its role in target gene transcription, ligand activated ERα has rapid effects on neuronal excitability via

second messenger systems, resulting in a range of cellular effects including changes in Ca^{2+} currents and activation of endothelial nitric oxide synthase (Kelly and Levin, 2001; Luconi *et al.*, 2002; Chen *et al.*, 1999). As changes in neuronal excitability have been implicated in migraine pathogenesis, we hypothesised that genetic variation in ERα may impact on expression or function, in turn influencing migraine susceptibility.

One particular ERα marker under investigation is a silent polymorphism in codon 594 of exon 8 and consists of a guanine to adenine change at nucleotide 2014. It was first described by Roodi *et al.*, (1985). The investigation was undertaken using a population based case-control approach. Due to past problems with non-replication of positive associations, we have also performed an additional study on an independent population based cohort using the same marker.

The human progesterone receptor gene is located on chromosome 11q22. It exists as 2 functionally distinct isoforms, PRA and PRB. PRB functions as a transcriptional activator of progesterone-responsive genes, while PRA is transcriptionally inactive and functions as a strong ligand-dependent repressor of steroid hormone receptor transcriptional activity (Giangrande *et al.*, 1997). Progesterone receptor expression is upregulated by estrogen and down-regulated by progesterone in most target tissues (Bouchard, 1999). The PR is found in various regions of the human brain including serotonin neurons (Lombardi *et al*, 2001; Bethea et al, 2002). Similar to ER, PR can undergo ligand-independent activation and is involved in various intracellular signalling pathways (Conni and Picard, 1999).

The PROGINS polymorphic *Alu* insertion is a 306 base pair insertion that occurs within intron G of the Progesterone receptor gene in some individuals. Although it does not occur within a coding region of the PR gene, it may have a deleterious effect on progesterone receptor expression, through recombination or mis-splicing (Rowe *et al.*, 1995; Donaldson, *et al.*, 2002). Kieback *et al.* (1995) and Wang-Gohrke *et al.* (2000). The PROGINS *Alu* insertion has been investigated for a possible role in breast cancer. In present study, it has been examined for a possible association with migraine due to its potential role in migraine pathogenesis.

The human AR is located on chromosome Xq11-12 and in humans is expressed in various organs including the brain in both males and females. It includes three major functional domains, the N-terminal domain, which is involved in transcriptional activation of target genes, coded for by exon 1, a cysteine rich DNA binding domain encoded by exons 2&3, and a hormone binding domain, encoded by exons 4-8 (Keller *et al.*, 1996).

A polyglutamine tract encoded by CAG repeats occurs in Exon 1 of the Androgen Receptor Gene. Expansion of this repeat is considered to have an inhibitory effect on transactivation function due to interaction of this region with various co-activators. Short fragments are associated with enhanced receptor function (Westberg *et al.*, 2001), while longer CAG repeats decrease AR activity. This reduced activity has been demonstrated to reduce negative feedback to the hypothalamus, resulting in increased serum androgen levels (Krithivas et al., 1999). Furthermore, abnormal expansions of poly-glutamine tracts in the central nervous system cause neurodegenerative diseases such as Huntingtons disease

and spinocerebellar ataxia type 1 (Chamberlain *et al*, 1994). It has been suggested that the effect of polyglutamine repeat length may be gene specific. The activity of the AR may be unaffected on genes that determine sexual differentiation, but compromised on genes necessary for normal neuronal function (Chamberlain *et al.*, 1994). Alleles of different sizes within the considered normal range of the AR CAG repeat have been associated with androgen dependent prostate-cancer (Yong *et al.*, 1998), and arterial vasoreactivity in males (Zitmann *et al.*, 2001). In this study the AR CAG repeat polymorphism will be examined for a potential association with migraine.

Materials and Methods

STUDY POPULATION

Research was approved by the Griffith University Ethics Committee for experimentation on human subjects. All participants of the study gave informed consent prior to participation. The case group consisted of 275 migraine sufferers who had been diagnosed with either migraine with aura (MA) or migraine without aura (MO) according to the widely accepted criteria in the International Headache Society guidelines. The control group of 275 individuals were matched for sex, age [+/- 5 years], and ethnicity [Caucasian] to avoid the potential bias of population stratification. Clinical characteristics of the case group appear in Table 1.

All participants provided a blood sample from which DNA was extracted by a modification of the salting out method used by Miller et al. (1988).

GENOTYPING

Genotyping for the ESRα G594A marker was undertaken by polymerase chain reaction (PCR) and restriction enzyme digestion. Oligonucleotide primers used were those previously described by Curran *et al* (1998). The 20 µl PCR reaction mix contained 50 ng genomic DNA, 0.25 µM of each primer, 1 x PCR buffer, 3.75 mM $MgCl_2$, 0.2mM dNTPs and DNA polymerase. Thermocycler conditions were 94 °C for 2 minutes 30 seconds, 5 cycles of 94 °C for 45 seconds, 69 °C for 1minute, and 72 °C for 2 minutes, followed by 30 cycles of 94 °C for 30 seconds, 67 °C for 30 seconds and 72 °C for 45 seconds, with a final step of 72 °C for 5 minutes. Following amplification, 10 µl of product was digested with BtgI overnight at 37 °C. After digestion, the product was loaded into a 5% Agarose gel stained with ethidium bromide and electrophoresed at 90V for 60 minutes. An undigested sample indicated presence of the 594A allele.

Genotyping for the PR progins insert marker was undertaken by polymerase chain reaction (PCR) and restriction enzyme digestion. Oligonucleotide primers used were those previously described by Lancaster *et al* (1998). The 20 µl PCR reaction mix contained 50 ng genomic DNA, 0.3 µM of each primer, 1 x PCR buffer, 2 mM $MgCl_2$, 0.2mM dNTPs and DNA polymerase. Thermocycler conditions were 94 °C for 5 minutes, followed by 30 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 30 seconds, with a final step of 72 °C for 2 minutes. Following amplification, 10 µl of product was loaded into a 2% Agarose gel stained with ethidium bromide and electrophoresed at 90V for 60 minutes.

Genotyping for the AR marker was undertaken by polymerase chain reaction (PCR) and capilliary electrophoresis using the ABI 310 Genescan™. Oligonucleotide primers used were those previously described by Sleddens *et al* (1992). The 15 µl PCR reaction mix contained 50 ng genomic DNA, 0.3 µM of each primer, Optimisation buffer H and DNA polymerase. Thermocycler conditions were 94 °C for 4 minutes, followed by 30 cycles of 94 °C for 60 seconds, 59 °C for 60 seconds and 72 °C for 30 seconds, with a final step of 72 °C for 2 minutes. Following amplification, Genotyping was carried out using the ABI 310 Genescan™ Genotyper computer software which converts the genescan sized peaks into genotype calls using macros.

STATISTICAL ANALYSIS

Genotype data and allele frequencies were compared between the two populations using chi-square analysis in the case of the ESRα and PR biallelic markers, and clump analysis in the case of the multiallelic AR gene trinucleotide repeat. We performed a power calculation according to known parameters of this study. Based on the rare allele frequency in controls being ~0.2, our power estimate analysis indicated that if the polymorphisms tested were to confer a 1.5-fold increase in relative risk of migraine, the case and control groups used in this study would be of sufficient size to have >85% power to detect an allelic association at the 0.01 level. It is important to acknowledge that these power estimates are not completely accurate because they do not factor in the effects of disease and marker parameters such as penetrances, trait-influencing allele frequency and distance from marker to trait-influencing locus. Genotype

frequencies were tested for Hardy-Weinberg Equilibrium to detect possible genotyping errors.

Results

Estrogen receptor results revealed a significant difference between migraineurs and the control group with regard to allele frequencies ($P = 0.003$) and genotype frequencies ($P = 0.008$). Results of comparisons between male and female migraineurs (allele frequency $P = 0.535$), male case and control groups (allele frequency $P = 0.034$), and female case and control groups (allele frequency $P = 0.032$) indicated that no significant gender difference was evident. Furthermore, no significant difference was seen in the comparison of the subgroups, MA and MO. Consequently, the significant association seen in the case-control analysis occurred similarly in both males and females, and MA and MO. Results indicated that individuals who carried the 594A allele were 1.8 times more likely to suffer from migraine [OR = 1.8, 95% CI = 1.2-2.6, $p = 0.003$] than those who did not carry this allele. See tables 2. and 3. Results of a follow-up study on an independent population-based cohort also revealed a significant difference between migraineurs and the control group with regard to allele frequencies ($P = 0.0004$) and genotype frequencies ($P = 0.0009$).

Statistical analysis of the PR results revealed a significant difference between the migraineur and control groups with regards to allele frequencies ($p = 0.015$) and genotype frequencies ($p = 0.033$). See tables 4. and 5.

Statistical analysis of the AR results revealed that no significant difference existed between the migraineur and control groups ($p = 0.24$). See tables 6. and 7.

Discussion

Migraine is considered to be a genetically complex disorder with familial aggregation. Approximately 50% of susceptibility is attributed to multiple genes while the balance is attributed to environmental factors. Although the genetic basis for migraine is largely unknown, investigations of various genes involved in key biological pathways have been undertaken. Results so far have been mixed, although this may be attributed in part to the heterogenous nature of the disorder. Current understanding of migraine is that a number of genes and/or environmental factors may each contribute to an individual's migraine susceptibility (Peroutka, 2002).

Steroid hormones, via their receptors, have many well-known effects on the central nervous system, particularly in relation to sexual differentiation and reproductive function. However, new information is emerging indicating a much wider range of effects than previously understood. Furthermore, these emerging effects have the potential to impact on factors involved in migraine pathogenesis. The present study considered that this role could be related to/or exacerbated by genetic variation in the estrogen receptor, progesterone receptor, and androgen receptor genes. Results of this study have indicated an association with the ESRα exon 8 polymorphism in two independent population based cohorts, as well as the progesterone receptor in a large cohort. To our knowledge, no investigations have been undertaken to explore this potential role, consequently no data exists to support or refute the findings presented in this study. On the other hand, significant evidence exists to support the hypothesis that both the estrogen and

androgen receptors may be integral players in mechanisms that are relevant to migraine pathogenesis, due to their hormone mediated roles.

Estradiol and progesterone can exert behavioural and electrophysiological effects by binding to neuronal membranes, in particular the central serotonergic and opioid neurons. This mechanism may lead to a disturbance of pain perception (Silberstein and Merriam, 2000). According to animal models, sex steroid hormones may also cause changes in regions involved in the neurovascular headache pathway (Marcus, 1995). Estrogen changes in the rat have been associated with altered mRNA expression in sensory neurons (Soharabji *et al.*, 1994). Additionally, estrogen injections appear to alter the size of the receptive area of the trigeminal mechanoreceptors in rats (Bereiter, 1980).

A further possible explanation for the role of estrogen receptor in migraine is its influence on calcium channels (Kelly & Levin, 2001). Due to the discovery of mutations in the CACNA1A calcium channel gene in familial hemiplegic migraine, a rare subtype of migraine, the role of calcium channels and calcium homeostasis in the pathogenesis of common migraine is highly likely. Calcium and other ion channels are significant factors in the mechanism of neurotransmitter release and cortical spreading depression (Edvinsson, 1999), thus impaired function of calcium channels could trigger an attack. Furthermore, an altered density of calcium channels would more easily result in excitation of the periaqueductal grey, raphe nuclei or locus coerulcus neurons that are considered to be in the region responsible for initiation of migraine attacks (Edvinsson, 1999).

L-type Ca^{2+} channels are one of the main pathways of intercellular calcium entry in the brain. Johnson *et al.* (1997) demonstrated in an animal model that the density of cardiac L-type Ca^{2+} channels is regulated by the estrogen receptor, while Mermelstein *et al.* (1996) demonstrated that estradiol reduces calcium currents in rat neostriatal neurons via a membrane receptor. A further consideration is that ER can mediate changes in vascular tone. Chen *et al.* (1998) demonstrated that ERα mediates the nongenomic activation of endothelial nitric oxide synthase, causing the rapid dilation of blood vessels. Furthermore, the observed response was evident at concentrations well below those found in normal cycling women (Chen *et al.*, 1998).

Obviously these hypotheses would require extensive analysis, as estrogen/ER action is understood to be tissue specific. Nevertheless they demonstrate potential mechanisms whereby genetic variation in the estrogen and progesterone receptors that alters function and/or expression can have a direct impact on mechanisms that are understood to be crucial to migraine pathogenesis.

Furthermore, as with all association studies, these results cannot elucidate a biological mechanism for this association, however they suggest that estrogen receptor variation may in fact be a further factor responsible for migraine susceptibility. Further research is necessary to uncover the mechanism involved in this susceptibility.

Table 1. Clinical characteristics of the case group

Gender	75 Male (27%) 200 Female (73%)
Family History	215 Yes (78.5%) 46 No (16.8%) 14 Unsure (4.7%)
Duration	Average 12-24 hours
Frequency	Average 1-2 per month
Age of Onset	Average 19.8 years

Table 2. Distribution of ESR Exon 8 Codon 594 ACG/ACA Polymorphism frequencies in migraineurs and controls

	Genotypes			N (alleles)	Alleles	
Group	11	12	22		1	2
Migraine (37%)	81 (36%)	120 (54%)	23 (10%)	448	282 (63%)	166
Male (39%)	18 (32%)	33 (58%)	6 (10%)	114	69 (61%)	45
Female (36%)	63 (38%)	87 (52%)	17 (10%)	334	213 (64%)	121
Control (28%)	112 (50%)	99 (44%)	13 (6%)	448	323 (72%)	125
Male (26%)	28 (49%)	28 (49%)	1 (2%)	114	84 (74%)	30
Female (28%)	84 (50%)	71 (43%)	12 (7%)	334	239 (72%)	95

11 = ACG/ACG, 12 = ACG/ACA, 22 = ACA/ACA

Table3. Chi-squared (χ^2) analysis of migraine groups for ESR Exon 8 Codon 594 ACG/ACA Polymorphism

	Frequency comparison	
Group	Genotypes	Alleles
Total Case V Control	$\chi^2 = 9.77$ $p = 0.008$	$\chi^2 = 8.56$ $p = 0.003$
Male V Female Migraineurs	$\chi^2 = 0.72$ $p = 0.699$	$\chi^2 = 0.38$ $p = 0.535$
Male Case V Control	$\chi^2 = 6.16$ $p = 0.046$	$\chi^2 = 4.47$ $p = 0.034$
Female Case V Control	$\chi^2 = 5.48$ $p = 0.064$	$\chi^2 = 4.63$ $p = 0.032$

Table4. Distribution of PgR PROGINS Polymorphism frequencies in migraineurs and controls

	Genotypes			N (alleles)	Alleles	
Group	11	12	22		1	2
Migraine 64(14%)	175 (75%)	55 (23%)	5 (2%)	232	404 (86%)	
Control (8%)	183 (85%)	33 (14%)	3 (1%)	219	396 (92%)	36

11 = No insert/No insert, 12 = No insert/PROGINS insert, 22 = PROGINS insert/PROGINS insert

Table 5. Chi-squared (X^2) analysis of migraine groups for PROGINS insert

Group	Frequency comparison	
	Genotypes	Alleles
Total Case V Control	$X^2 = 6.9$ $p = 0.033$	$X^2 = 6.4$ $p = 0.015$

Table 6. Distribution of AR CAG repeats

	Allele counts																	total
Allele	171	174	177	180	183	186	189	192	195	198	201	204	207	210	213	217	222	
No Rpts	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	
Cases	1	4	2	2	16	35	51	57	67	37	51	26	19	6	6	4	1	385
Male 61	0	1	0	0	5	5	7	8	9	10	5	1	5	1	2	1	1	
Female	1	3	2	2	11	30	44	49	58	27	46	25	14	5	4	3	0	324
Controls 319	0	1	0	3	9	28	53	62	51	27	36	23	9	15	1	1	0	
Male 45	0	0	0	0	1	4	6	11	5	4	4	5	1	3	0	1	0	
Female 274	0	1	0	3	8	24	47	51	46	23	32	18	8	12	1	0	0	

Table 7. Clump analysis of migraine groups for AR CAG repeat

	Frequency comparison	
Group	T1	T4
Total Case V Control	22.39 $p = 0.10$	10.5 $p = 0.24$
Male V Female Migraineurs	20.98 $p = 0.19$	14.12 $p = 0.09$
Male Case V Control	15.42 $p = 0.27$	9.38 $p = 0.34$
Female Case V Control	16.15 $p = 0.37$	7.11 $p = 0.55$

Throughout this specification, the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. Various changes and modifications may be made to the embodiments described and illustrated herein without departing from the broad spirit and scope of the invention.

All computer programs, algorithms, patent and scientific literature referred to in this specification are incorporated herein by reference in their entirety.

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